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DESIGN SYNTHESIS AND STUDY OF CATALYSTS FOR
ORGANOPHOSPHATE ESTER HYDROLYSIS(U) ALBERTA UNIV
EDMONTON DEPT OF CHEMISTRY R S BROWN JUL 85

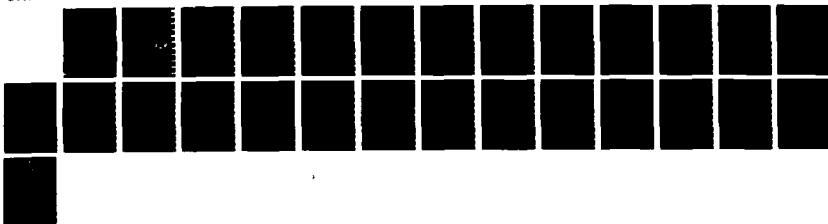
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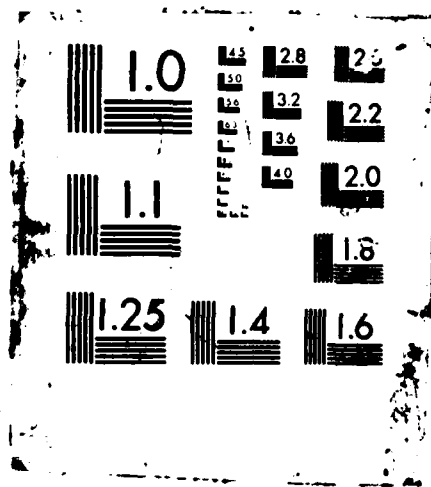
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Design, Synthesis and Study of Catalysts for Organophosphate Ester Hydrolysis		5. TYPE OF REPORT & PERIOD COVERED Annual - March 15, 1984 to March 14, 1985
7. AUTHOR(s) Robert S. Brown		6. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Chemistry University of Alberta Edmonton, Alberta, Canada, T6G 2G2		8. CONTRACT OR GRANT NUMBER(s) DAMD17-83-C-3091
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Fort Detrick, Frederick, Maryland 21701-5012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62734A.3M162734A875.AJ.090
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE July 1985
		13. NUMBER OF PAGES
		15. SECURITY CLASS. (of this report) Stat
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 26, if different from Report) DTIC SELECTED APR 14 1987		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Alkaline phosphatase, models, catalysis, organophosphate ester, hydrolysis, metal ion		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The hydrolyses of <u>tris-p-nitrophenylphosphate</u> (5), <u>tris-2-pyridylphosphate</u> (6) and <u>ethyl-p-nitrophenylphosphonate</u> (7) catalyzed by two <u>tris-imidazol-yl-</u> <u>phosphine-Co²⁺</u> complexes (Co ²⁺ :3 and Co ²⁺ :4) were studied as a function of pH. In the hydrolyses of both 6 and 7, Co ²⁺ :3 (which contains no hydroxyethyl pendant) appears to act as its anionic form, generated by ionization of a Co ²⁺ :4 bound H ₂ O having a pK _a of 6.8 at 37°C in 80% ethanol-H ₂ O. On the other hand, Co ²⁺ :4 OVER...		

20. ABSTRACT continued:

cont'd → (which possesses a hydroxyethyl pendant) catalyzes the hydrolyses of 6 and 7 with an activity that increases linearly with pH up to at least pH 8.3. Above this pH, precipitation of $\text{Co}^{2+}(\text{OH})_2 \cdot x$ becomes a problem. In the latter case, the active form of $\text{Co}^{2+}:4$ has a pK_a in excess of 8.3 that apparently obtains from ionization of the Co^{2+} -associated hydroxyethyl group. Evidence is presented that $\text{Co}^{2+}:4$ functions as a general base catalyst in the hydrolysis of 6.

(Keywords:)

AD _____

Design, Synthesis and Study of Catalysts
for Organophosphate Ester Hydrolysis

Annual Report

Robert S. Brown

July 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3091

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Summary

This Annual Report details the work carried out during the contracting year (March 1984 - March 1985) under Contract DAMD17-83-C-3091. Section I describes very briefly the approach taken, namely, to design and synthesize catalysts for phosphate ester hydrolyses which are modelled after carbonic anhydrase (CA) and alkaline phosphatase (APase). Section II describes the kinetic studies undertaken since the last contracting year. We have investigated in considerable detail the hydrolysis of ethyl-p-nitrophenylmethylphosphonate (7), tris-p-nitrophenylphosphate (5), and tris-2-pyridylphosphate (6) catalysed by the Co^{2+} -complexes of two tris-imidazolylphosphines (3 and 4). In this study we found that both Co^{2+} -complexes facilitated the hydrolysis of 5 to 7, but they do so by different mechanisms. While 3: Co^{2+} reacts via a metal-coordinated OH_2 group having a pK_a of ~ 8.0 at 37°C in 80% ethanol- H_2O , 4: Co^{2+} apparently reacts via the involvement of a metal-associated hydroxyethyl pendant.

Foreword

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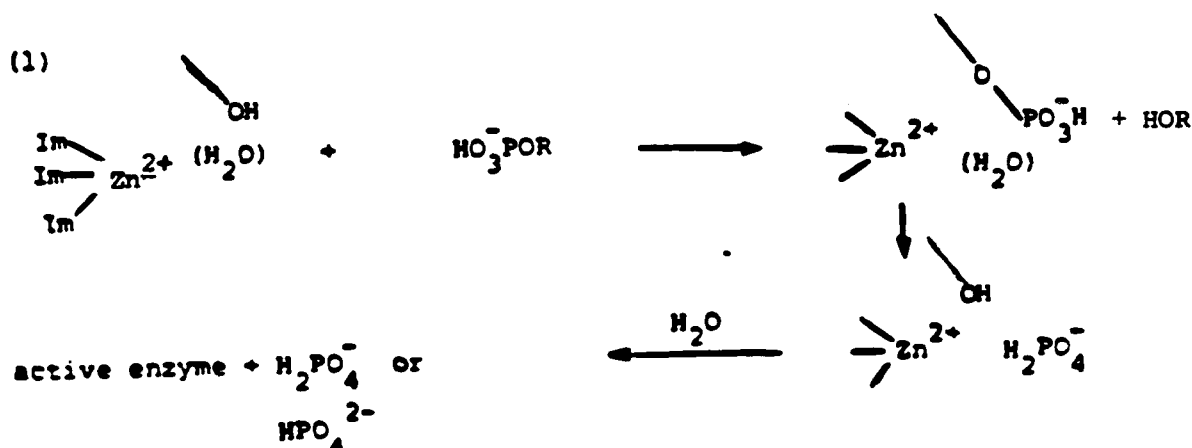
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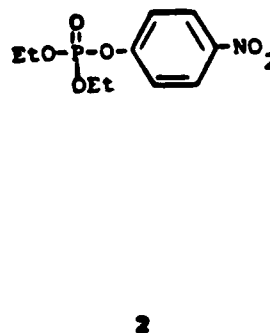
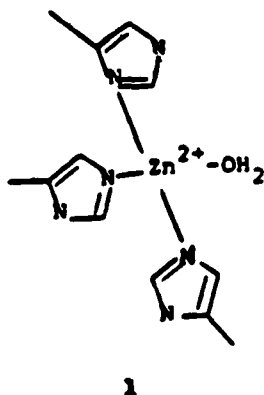
I. Statement of Project

(a) Carbonic Anhydrase and Alkaline Phosphatase Models as Catalysts for Hydrolysis of Phosphate Esters.

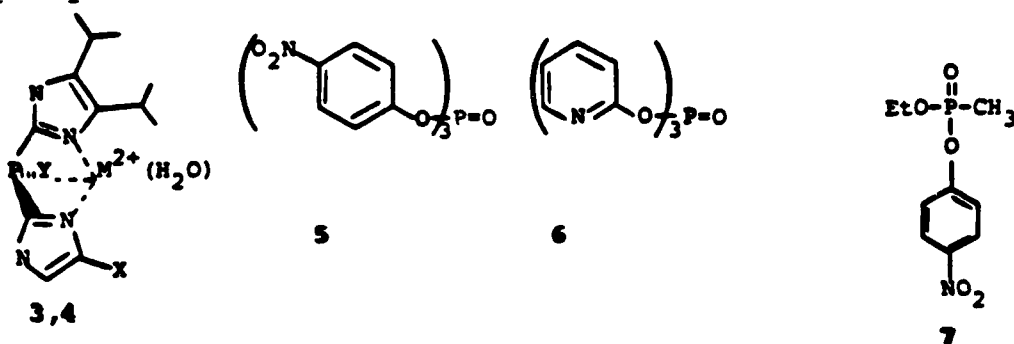
Alkaline phosphatases (APases) are Zn(II)- and Mg(II)-containing metalloenzymes found in virtually every living organism which catalyse the hydrolyses of phosphate monoesters (ROPO_3H_2) of wide structural variation.¹ It now seems reasonably certain that the active site catalytic groups consist of two Zn(II) ions ~ 5Å apart respectively coordinated by three histidine residues and a histidine and two aspartate residues.^{1,2} The remaining ligand positions are occupied by H_2O . Also in the active site is an essential serine OH group which becomes transiently phosphorylated during the catalytic cycle. Although the exact mechanism of action is unknown, a minimum reaction scheme can be drawn as in equation 1.



Mechanistically, the role of the Zn^{2+} is, first, to activate the serine OH sufficiently to enable phosphoryl transfer to it, and second, to dephosphorylate that residue, probably by delivering a Zn^{2+} -bound H_2O or OH^- group. The parallel with the inhibition of acetylcholinesterase (AChE) by organophosphates is close, except that AChE has no internal mechanism by which it can hydrolyse the phosphoryl (phosphonyl) serine, since there is no Zn^{2+} at the active site.



Carbonic anhydrase (CA) is a metalloenzyme with an active site consisting of a Zn^{2+} -ion coordinated to three histidine imidazole (Im) units as in 1.³ In addition to its normal physiological function of catalysing the interconversion of HCO_3^- and CO_2 , CA has been shown to have oxonase activity in that it catalyses the hydrolysis of a dialkyl aryl phosphate triester analogue of paraoxon (2).⁴ Given the above, and the fact that a number of less-well-characterized enzymes have been shown to exhibit oxonase activities,⁵ it is of interest to know whether smaller, biomimetic models for the CA and APase active sites (3 and 4)⁶ would act as catalysts in facilitating the hydrolysis of some selected phosphate triesters such as 5⁷ and 6⁸ and a phosphonate 7.⁹



3: X = H, Y = 4,5-diisopropylimidazol-2-yl

4: X = $\text{CH}_2\text{CH}_2\text{OH}$, Y = 4,5-diisopropylimidazol-2-yl

The latter compound can be taken as a model for the phosphoryl inhibited serine OH group in AChE.

(b) Overview of Catalysis of Phosphate (Phosphonate) Ester Hydrolyses

Considerable effort has been expended to define conditions under which the hydrolyses of phosphate and phosphonate esters are catalysed. Much of the work¹⁰⁻¹³ centers on the use of micelles which contain functional groups such as Im¹⁰, oximate¹², or alcohols.¹³ Im-containing surfactants¹⁰ have been found to hydrolyse phosphate triesters by nucleophilic and general base mechanisms. Oximate¹² and alcohol(ate) surfactants attack triesters and aryl alkyl phosphate diesters nucleophilically, the main role of the tetraalkylammonium surfactant being to provide a positively charged medium in which the reaction can occur. Generally these studies used highly alkaline conditions.

Under non-micellar conditions, Macomber¹⁴ has investigated the catalysis afforded by Im on the hydrolysis of oxa-phospholene esters (8). Although Im acts as a nucleophile toward tetra-benzylpyrophosphate (9)¹⁵, its role with 8 is that of a general base.¹⁴ When present in 2.4-fold excess over 8, Im affords a 50-fold acceleration over the spontaneous hydrolysis rate.

Kinetics were monitored by observing the rate of appearance of *p*-nitrophenolate at 400 nm for 5 and 7 or 2-pyridinol at 305 nm for 6 in 80% ethanol-H₂O buffered solutions held at 37.1±0.2°C or 25.7±0.2°C, as previously described.²⁰ Buffers (0.047 M, μ = 0.047 NaClO₄) were CHES (cyclohexylaminoethanesulfonic acid), pH 7.5-8.3, or MOPS (morpholinoethanesulfonic acid), pH 5.4-7.2). pH values were measured with a radiometer GK 2322 C combination electrode immersed directly into the solution before and after each reaction and were not corrected for the organic solvent fraction. Reactions were initiated by injecting known quantities of substrate (5-10 x 10⁻³ M tetrahydrofuran [THF] stock solutions of 5-7), ligand (0.025 M ethanol stock) and CoCl₂ (0.1314 M H₂O stock standardized by EDTA titration²¹) into 3.0 mL thermally equilibrated buffer. Pseudo-first-order rate constants were evaluated by fitting the absorbance vs. time data to a standard exponential model ($A_t = A_{t_\infty} + (A_{t_0} - A_{t_\infty}) e^{-kt}$) by a non-linear least squares treatment. In all cases the reactions were followed in duplicate or triplicate to at least 80% completion and displayed good pseudo-first-order kinetics. Reported values are averages of two to three determinations and have a precision of better than 5%.

Product studies were conducted by comparing the uv-vis spectra obtained after reaction with a mixture comprised of the authentic catalytic components and expected hydrolytic products, (ethylmethylphosphonate + *p*-nitrophenol in the case of 7, bis-2-pyridylphosphate + 2-pyridinol in the case of 6, and bis-*p*-nitrophenylphosphate + *p*-nitrophenol in the case of 5). In all cases the spectra obtained after reaction were identical with those of the anticipated product mixtures. Control experiments established that for the latter two cases only one leaving group is cleaved from the substrate and hence the initially formed phosphate products are stable to further hydrolysis.

(b) Results

i. Hydrolyses of 5 and 7.

The hydroxide-dependent rate constants for hydrolysis of 5 and 7 (H₂O, 25°C) are reported to be 11.2 M⁻¹s⁻¹ and 0.04 M⁻¹s⁻¹, respectively.^{7a} In 80% ethanol-H₂O (this medium required for ligand solubility) at 25.7°C, the spontaneous pseudo-first-order rate constants for hydrolysis of 5 at pH 7.5 and pH 7.85 (MOPS) are 5.12 x 10⁻⁴s⁻¹ and 7.19 x 10⁻⁴s⁻¹, respectively (faster than that reported in 1:1 acetone/H₂O; 1 x 10⁻⁵s⁻¹, pH 7.5, 25°C^{7a}), while those of phosphonate 7 are too slow to be measured. Neither Co²⁺ nor ligand 3 or 4 alone at 5 x 10⁻⁴ M facilitates the decomposition of either substrate. However, equimolar Co²⁺ and 4 produce accelerations, the second-order catalytic rate constants being given in Table 1. Previous studies⁶ have shown that ligands 3 and 4 have high affinities for Co²⁺ such that at

Table 1. Hydrolysis of tris-p-nitrophenylphosphate (5) and ethyl-p-nitrophenylmethylphosphonate (7) catalysed by $\text{Co}^{2+}:4$ at 25.7°C.^{a,b}

pH	$k_5^{\text{cat}} \times 10^2 (\text{M}^{-1}\text{s}^{-1})^c$	$k_7^{\text{cat}} \times 10^2 (\text{M}^{-1}\text{s}^{-1})^c$
7.50	6.4	1.4
7.85	9.8	2.7
7.95	-	3.4
8.20	-	8.6
8.30	-	10.2

- a. 80% ethanol- H_2O as in reference 8d; 0.047 M CHES buffer; $\mu = 0.047 \text{ NaClO}_4$.
b. 0.05 mM 5 and 7; 0.5-2.0 mM $\text{Co}^{2+}:4$; followed at 400 nm.
c. Evaluated from the slopes of plots of k_{obs} vs $[\text{Co}^{2+}:4]$; according to equation $k_{\text{obs}} = \text{intercept} + k_x^{\text{cat}}[\text{Co}^{2+}:4]$; $r > 0.99$ for at least 5 concentrations.

the concentrations employed, the complexes are >95% formed.* This is also verified by the fact that plots of k_{obs} vs. $[\text{Co}^{2+}:4]$ are linear and show no upward curvature between 0.5 and 2.0 mM in added components. The linearity also argues against a pre-equilibrium formation of a phosphate or phosphonate: $\text{Co}^{2+}:4$ ternary complex at these concentrations. The fact that the catalytic second-order rate constants presented in Table 1 increase with pH indicates that the active form of $\text{Co}^{2+}:4$ is in some way dependent on $[\text{OH}^-]$, although from these data the exact nature of the active form is uncertain.

Unfortunately, the relative activities of $\text{Co}^{2+}:4$ and $\text{Co}^{2+}:3$ toward substrates 5 and 7 cannot be compared at 25°C, since solutions of Co^{2+} and 3 become turbid during the course of the reactions. To make such a comparison, a temperature of 37.1°C was used for further hydrolysis studies of 7. Given in Table 2 are the pseudo-first-order rate constants for the hydrolysis of 0.05 mM 7 observed at 37°C in the presence of 0.5 mM $\text{Co}^{2+}:3$ or $\text{Co}^{2+}:4$. From these data it can be observed that $\text{Co}^{2+}:4$ is about 3-7-fold more active than is $\text{Co}^{2+}:3$, the difference in activities being greater at higher pH. It is also evident from Fig. 1 that the activity of $\text{Co}^{2+}:3$ levels off at about pH 8.0, indicating an ionization event, while the activity of $\text{Co}^{2+}:4$ continues to increase with pH in a first-order fashion.

*The $\text{pK}_{\text{Co}^{2+}}$ values (defined in the direction of dissociation) for $\text{Co}^{2+}:3$ and $\text{Co}^{2+}:4$ in 80% ethanol H_2O are 7.7 and 5.7, respectively.⁶ The Eadie-Hofstee plot for the decomposition of 6 as a function of $[\text{Co}^{2+}]$ yields an equivalent pK_{eq} for $6:\text{Co}^{2+}$ of 2.2.

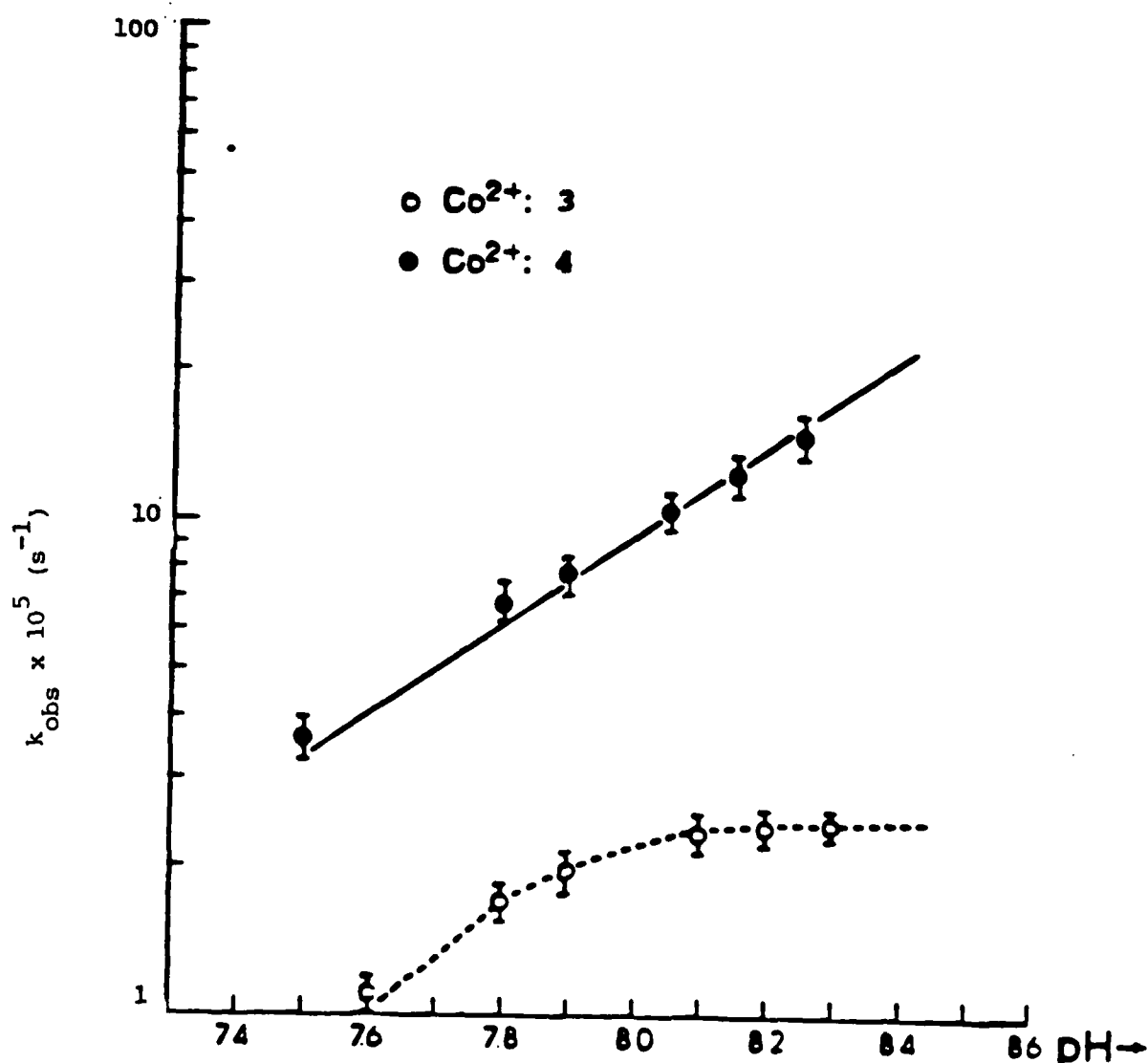


Figure 1

Plot of $k_{\text{obs}} \times 10^5$ for the hydrolysis of $5 \times 10^{-5}\text{M}$ phosphonate 7 in the presence of $5 \times 10^{-4}\text{M}$ $\text{Co}^{2+}:3$ (○) and $\text{Co}^{2+}:4$ (●). $T = 37.1^\circ\text{C}$, 0.047μ , CHES buffers, $\mu = 0.047$. NaClO_4 , 80% ethanol- H_2O . Straight line drawn through $\text{Co}^{2+}:4$ data is first order in $[\text{OH}^-]$. Data from Table 2 with error limits being $< \pm 5\%$ of the value as determined by averages of duplicate experiments.

Table 2. Pseudo-first-order rate constants (k_{obs}) for the hydrolysis of 7 in the presence of Co^{2+} :3 and Co^{2+} :4 at 37.1°C .^{a,b}

pH	$k_{\text{obs}}(\text{Co}^{2+}:3) \times 10^5 \text{s}^{-1}^c$	$k_{\text{obs}}(\text{Co}^{2+}:4) \times 10^5 \text{s}^{-1}^c$
7.50	-	3.64
7.80	1.70	6.75
7.90	1.95	7.80
8.10	2.35	10.6
8.20	2.41	13.5
8.30	2.45	15.1

a. 80% ethanol- H_2O ; 0.047 M CHES; $\mu = 0.047 \text{ NaClO}_4$.

b. Followed at 400 nm.

c. 0.5 mM Co^{2+} :3 or Co^{2+} :4; 0.05 mM 7; values are averages of at least duplicate measurements.

ii. Hydrolysis of tris-2-pyridylphosphate (6).

Uncatalysed, the pseudo-first-order rate constant for hydrolysis of 6 between pH 6.0 and 8.2 varies by only a factor of 3 from 5.27 to $18.7 \times 10^{-6} \text{s}^{-1}$, suggestive of a dominant water term and the onset of a small, OH^- -dependent term at the high pH end of this region. Addition of a 10-fold excess of Co^{2+} (0.5 mM) accelerates the reaction by nearly 100-fold at all pH's, these data being given in Table 3 along with data for the

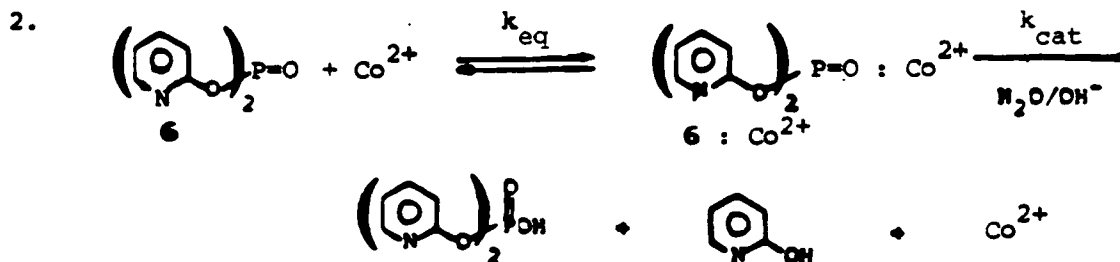
Table 3. Pseudo-first-order rate constants (k_{obs}) for the hydrolysis of 0.05 mM tris-2-pyridylphosphate (6) in the presence of Co^{2+} , Co^{2+} :3, and Co^{2+} :4.

$k_{\text{obs}} \times 10^4 \text{s}^{-1}$			
pH	Co^{2+}^b	$\text{Co}^{2+}:3^c$	$\text{Co}^{2+}:4^c$
5.40	4.52	-	-
5.80	4.19	-	-
6.00	4.55	3.82	2.66
6.40	4.37	4.55	3.17
6.80	5.02	5.93	3.44
7.10	6.31	8.28	5.61
7.45	13.1	19.0	13.7
7.85	20.0	30.4	27.0
8.10	-	38.4	-
8.15	-	41.1	68.4

- a. $T = 37.1^\circ$; 0.047 M MOPS (pH 5.4-7.1) or 0.047 M CHES (pH 7.1); $\mu = 0.047$ NaClO₄; 80% ethanol H₂O; followed at 305 nm; uncertainties $\pm 5\%$.
- b. 0.493 mM CoCl₂.
- c. 0.484 mM complex.

observed in the presence of Co²⁺:3 and Co²⁺:4. The latter data are displayed in Fig. 2 and show that the catalysis exhibited by Co²⁺:3 levels off above pH 8 while Co²⁺:4 catalysis increases linearly from pH 7 to 8.2. Unfortunately, precipitation is observed above pH 8.2, which prevents complete definition of the pH profile. Ligand alone does not accelerate the reaction.

Plots of k_{obs} against [Co²⁺] alone show definite signs of curvature between 0.5 and 5.5 mM Co²⁺ suggesting a saturation phenomenon as in equation 2. At a given pH, an Eadie-Hofstee plot of k_{obs} against $k_{\text{obs}}/[\text{Co}^{2+}]$ yields a straight line with



intercept reaction k_{cat} and a slope $-K_{\text{eq}}$, the latter value being defined as the dissociation constant of $\text{6} : \text{Co}^{2+}$. At pH 6.4, the derived constants are $k_{\text{cat}} = (4.67 \pm .17) \times 10^{-3} \text{ s}^{-1}$ and $K_{\text{eq}} = (4.68 \pm .41) \times 10^{-3} \text{ M}$ ($r = 0.98$, 8 data), while at pH 7.15, the values are $(7.32 \pm .22) \times 10^{-3} \text{ s}^{-1}$ and $(5.13 \pm .37) \times 10^{-3} \text{ M}$ ($r = 0.98$, 9 data). These data indicate that (within experimental error) the dissociation constant for 6 and Co²⁺ is equivalent at the two pH's, while the catalysed rate constant increases with pH, indicating a dependence on [OH⁻].

Hydrolysis of 6 is also promoted by the Co²⁺ complexes of 3 and 4, although from the data given in Table 3, the two complexes are approximately as reactive as Co²⁺ alone. Since 3 and 4 bind Co²⁺ at least 10³ more tightly than does 6*, the great bulk of Co²⁺ will be coordinated to those ligands, leaving little free metal in solution. Hence, whatever acceleration occurs in hydrolysis must result from the 3:Co²⁺ or 4:Co²⁺ complexes. Plots of k_{obs} vs. [4:Co²⁺] between 0.4 and 5.0 mM at pH 6.4 and 7.15 yield straight lines with slopes of $0.58 \pm .07 \text{ M}^{-1} \text{ s}^{-1}$ and $2.0 \pm .15 \text{ M}^{-1} \text{ s}^{-1}$, indicating that the second-order catalytic rate constants increase with pH in (within experimental error) a first-order fashion. No evidence of saturation behavior is evident.

(c) Discussion

Neutral phosphate and phosphonate esters at pH values above 7 generally decompose by pathways dependent on the concentration of anionic nucleophiles.^{7,22} Hydroxide is a reasonably good nucleophile, but at pH values approaching neutrality, its concentration is so low that the pseudo-first-order rate constants for hydrolysis of esters such as 5 or 7 are 10^{-6} - 10^{-8} s⁻¹ in H₂O.^{22a}

In a variety of systems, it has been shown that the pK_a of an M^{2+} -coordinated H₂O is drastically reduced but it retains sufficient nucleophilicity to attack electrophilic centers.^{3,23} Several studies have shown that M^{x+} -coordinated hydroxyalkyl or oxime groups also have reduced pK_a values and retain sufficient nucleophilicity as their anions to attack esters, CO₂ and phosphate esters.²⁴ Coordination of metal ions to normally hydrolysis-resistant anionic phosphates and polyphosphates has been reported to lead to large accelerations in hydrolysis by mechanisms which could involve a combination of Lewis acid charge neutralization, intramolecular delivery of M^{x+} -OH⁻, and/or electrostatic stabilization of the leaving group.^{23a,25}

Ligand 3, when coordinated to Zn²⁺ or Co²⁺, appears to be a reasonable model for the active site of carbonic anhydrase, while M^{2+} -coordinated 4 is a model for a portion of the active site of APase.* Earlier studies have shown that Co²⁺:3 and Co²⁺:4 both undergo ionization of a Co²⁺-OH₂ with a kinetic pK_a of 7.6-7.8 at 25.0°C in 80% ethanol-H₂O to produce a Co²⁺-OH⁻ unit that acts as a bimolecular catalyst in promoting the hydrolysis of *p*-nitrophenyl picolinate (pNPP) (equation 3).^{6b} No evidence of the formation of a ternary pNPP:Co²⁺:3 or pNPP:Co²⁺:4 complex is evident. This study^{6b} also indicated that the hydroxyethyl group in Co²⁺:4 is of no catalytic benefit in pNPP hydrolysis, even though in related examples²⁴ a hydroxyalkyl pendant is reported to act as a nucleophile. The major catalytic advantage of Co²⁺:3 and Co²⁺:4 over Co²⁺(H₂O)_x is two-fold: first, to reduce the pK_a of a coordinated H₂O and second, to bind Co²⁺ tightly enough to

*The active site of CA has been determined by X-ray crystallography to consist of an essential Zn²⁺-ion coordinated in a tetrahedral manner to three protein-based histidine Im units.³ The active site of AP, while not yet completely elucidated by X-ray crystallographic analysis, appears to consist in part of an essential Zn²⁺-ion coordinated to at least three histidine Im units.¹ Also in the active site is an essential serine OH group, which during the hydrolytic event becomes transiently phosphorylated. The latest structural determinations indicate that the active site is dinuclear in Zn²⁺, the second Zn²⁺ being bound by 1 His Im and two oxygen-containing ligands.

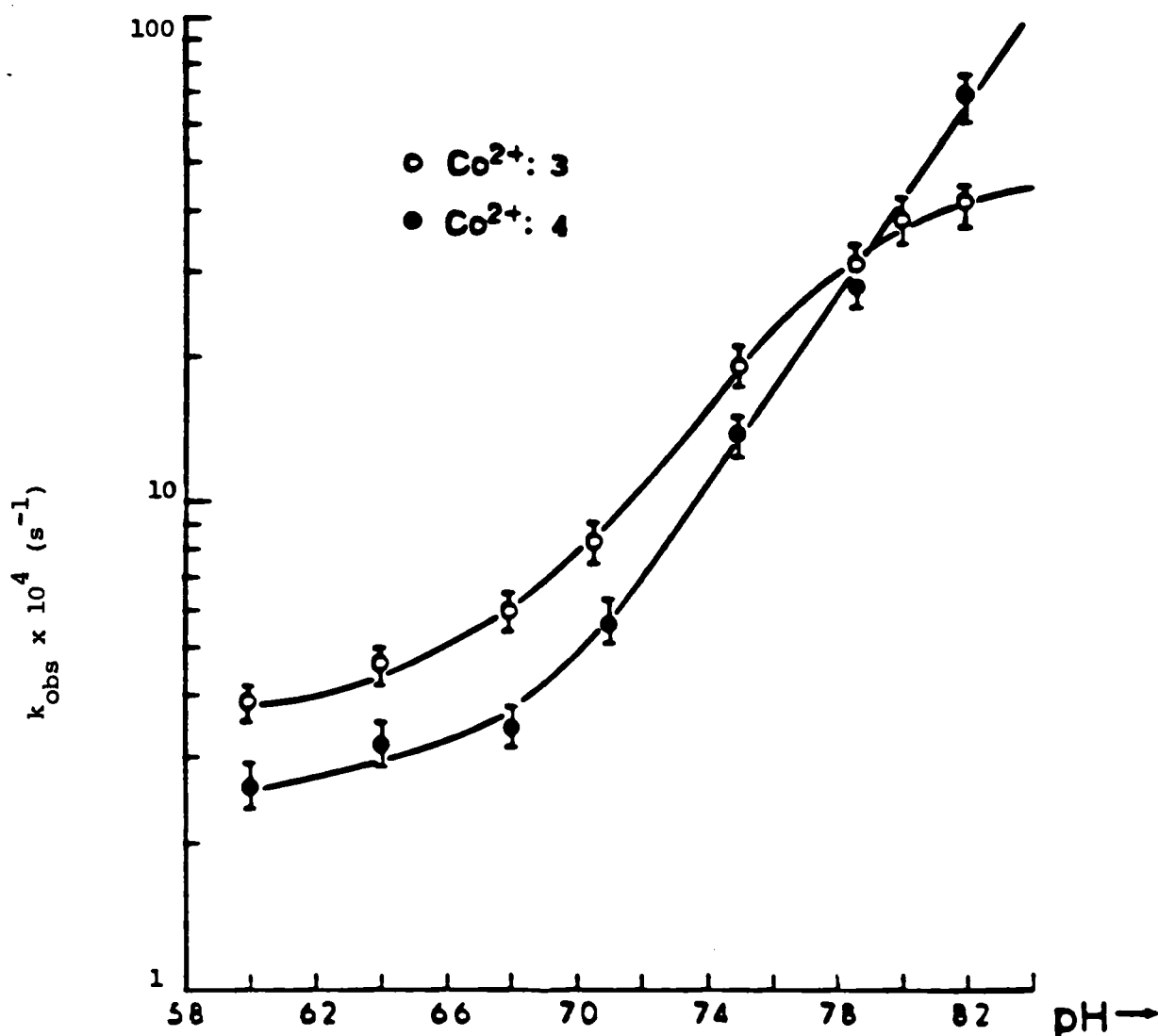
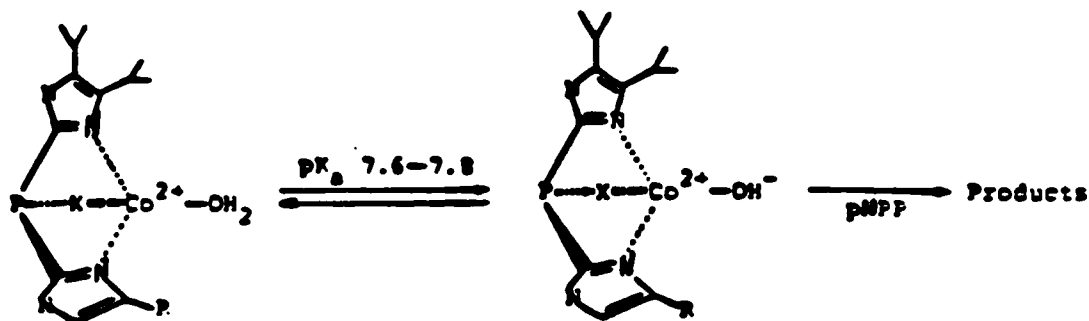


Figure 2

Plot of $k_{\text{obs}} \times 10^4$ for the hydrolysis of $5 \times 10^{-5} \text{ M}$ tris-2-pyridylphosphate (6) in the presence of $4.8 \times 10^{-4} \text{ M Co}^{2+}:3$ (\circ) and $\text{Co}^{2+}:4$ (\bullet). $T = 37.1^\circ \text{C}$, 0.047 M MOPS or CHES buffers, $\mu = 0.047 \text{ NaClO}_4$, 80% ethanol- H_2O . Error limits $< \pm 5\%$ of value determined by average of duplicate experiments.

prevent precipitation of $\text{Co}^{2+}(\text{OH}^-)_x$ during the course of the reaction.

3.



$\text{Co}^{2+}:3$: $\text{R} = \text{H}$; $\text{X} = \text{diisopropylimidazole}$

$\text{Co}^{2+}:4$: $\text{R} = \text{CH}_2\text{CH}_2\text{OH}$; $\text{X} = \text{diisopropylimidazole}$

Toward phosphonate 7 at 37.1°C , both $\text{Co}^{2+}:3$ and $\text{Co}^{2+}:4$ exhibit activity, but the plots given in Fig. 1 indicate that they behave differently. $\text{Co}^{2+}:3$ shows an increasing activity with pH below pH 7.8 and levels off above 8, indicating the participation of a deprotonated form of the complex. We tentatively ascribe this to an ionizing $\text{Co}^{2+}:3$ -bound H_2O with a $\text{pK}_a \sim 8.0$. This value is somewhat higher than the 7.6-7.8 noted for the same ionization in the catalysed hydrolysis of pNPP^{6b} and may be attributable to the slightly different conditions and 12°C higher temperature used for the present study. Admittedly, the pH range covered here is not wide enough to make a definite conclusion concerning the pK_a . This is because the reactions are quite slow at pH values lower than 7.4, where they are complicated by ligand instability while at pH values higher than 8.3, precipitation becomes a problem.

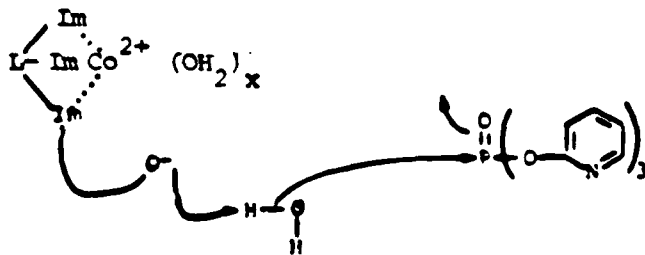
Apparently $\text{Co}^{2+}:4$ behaves differently from $\text{Co}^{2+}:3$, since the former's activity toward phosphonate 7 continues to increase with pH with no evidence of curvature. This implies that a basic form of $\text{Co}^{2+}:4$ is active but that the ionizing group pK_a is higher than that of the Co^{2+} -bound H_2O , which was shown in a previous study to be 7.6-7.8 at 25°C .^{6b} By analogy to related systems bearing a pendant hydroxyalkyl group,²⁴ we ascribe the ionization to the Co^{2+} -associated hydroxyethyl group which should have a higher pK_a than 8. Whereas the activity of $\text{Co}^{2+}:4$ toward pNPP involves the metal-associated H_2O , reaction toward these phosphorus centers apparently involves the hydroxyethyl pendant. Plots of k_{obs} vs. $[\text{Co}^{2+}:4]$ at each pH are linear and do not indicate formation of a ternary 7: $\text{Co}^{2+}:4$ complex.

Hydrolysis of phosphate 6 is markedly catalysed by Co^{2+} and saturation behavior is observed in plots of k_{obs} vs. $[\text{Co}^{2+}]$. This indicates pre-equilibrium formation of a 6: Co^{2+} complex, which suffers attack by H_2O and or OH^- as in equation 2. From

the Eadie-Hofstee plot, the k_{cat} values for the decomposition of 6:Co²⁺ at pH 6.4 and 7.15 are $4.67 \times 10^{-3} s^{-1}$ and $7.32 \times 10^{-3} s^{-1}$ and show a slight but not first-order dependence on [OH⁻]. The coordinated Co²⁺ likely acts both as a Lewis acid to facilitate attack on the central P and to stabilize the departing alkoxy-pyridine, both processes having precedent.^{24,25}

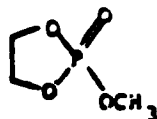
Co²⁺:3 and Co²⁺:4 also catalyze the solvolysis of 6 in a way which increases with pH (Fig. 2). However, the plot for Co²⁺:3 levels off, indicative of an active species having a $pK_a \sim 8$, while that for Co²⁺:4 continues to increase linearly with pH. The implication is that the activity for the former is associated with 3:Co²⁺-OH⁻, while the different behavior of Co²⁺:4 is tied to the presence of the hydroxyethyl group, which in its basic form is promoting the solvolysis of 6. Plots of k_{obs} vs. [Co²⁺:4] show a strict linearity, the slopes of which increase with pH and give no evidence of saturation behavior.

All of the above points to the fact that the Co²⁺:4 behaves differently from Co²⁺:3 in terms of its relative propensity to attack the neutral phosphates and phosphonate studied here. The basic ligand structure in Co²⁺:3 and Co²⁺:4 is the same with respect to numbers, orientation, and types of Im's. Earlier studies^{6b} have revealed that the pK_a value of the Co²⁺-coordinated H₂O units is the same for both complexes. Hence, it seems reasonable that the different behavior of the two toward phosphate ester hydrolysis is ascribable to the anionic form of the hydroxyethyl pendant, which apparently has a pK_a in excess of 8.3 in the medium used. The pendant could act either as a nucleophile or general base, although for several reasons we favor the latter role. First, although the bulk of the studies reported here employed an excess of complex relative to phosphate 6 or phosphonate 7, when equimolar concentrations ($5 \times 10^{-4} M$) of Co²⁺:4 and 6 or 7 were employed, the production of *p*-nitrophenoxide exhibited clean first order kinetics. Second, product studies revealed that hydrolysis of an equivalent of 6 catalysed by Co²⁺:4 yielded only one equivalent of 2-pyridinol. Dipyriddyphosphate is not hydrolysed under the conditions employed here nor is its hydrolysis catalysed visibly by Co²⁺ or either complex. These two observations suggest strongly that the role of the anionic hydroxyethyl pendant in Co²⁺:4 is that of a general base as in 13.

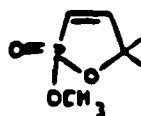


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General base roles are well documented, for example, in the hydrolyses of phosphate 14¹⁴ or phosphonate 15.¹⁵ Were $\text{Co}^{2+}:4$ to

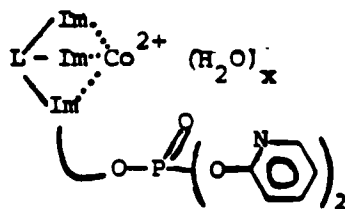


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behave as a nucleophile toward 6, intermediate 16 would be formed. To satisfy the kinetics and the observation that hydrolysis of 6 produces only one molecule of 2-pyridinol



16 L = 3

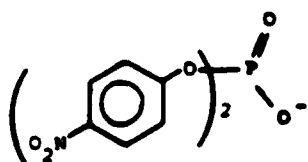
would require that 16 hydrolyse at least 10 times faster than it is formed (to maintain a constant $[\text{Co}^{2+}:4]$) by a pathway that involves exclusive cleavage of the $\text{L}:\text{Co}^{2+}\text{O}-\text{P}$ bond. However, it is anticipated that cleavage of a 2-pyridinol unit from 16 would be a much more rapid process, since the heterocycle is a far better leaving group than is the hydroxyethyl group of $\text{Co}^{2+}:4$. Unfortunately, synthesis of the putative intermediate 16 proved impossible in our hands, yielding only complex mixtures of what appeared to be a cyclic phosphate having both the N and O of the hydroxyethyl Im unit covalently attached to phosphorus.

The above account provides evidence that complexes such as $\text{Co}^{2+}:4$ and $\text{Co}^{2+}:3$ can be reasonably effective catalysts in promoting the hydrolysis of selected neutral phosphonate and phosphate substrates. In 80% ethanol- H_2O at 25°C , the apparent second order rate constant of $\text{Co}^{2+}:4$ toward phosphonate 7 increases linearly with pH up to at least pH 8.3, where its value of $k_{\text{cat}} = 10.2 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ exceeds that of OH^- ($k_{\text{OH}^-} = 3.9 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$), even though the active form of $\text{Co}^{2+}:4$ is not completely generated. This value compares favorably with the apparent second-order rate constants for some Co^{3+} chelates¹⁷ and aldoximates¹⁹ toward 7, recently published by Kenley et al. For example, in H_2O at 25° , pH 7.6, the apparent k_{cat} terms for complex 11 and 2-PAM (12) are $5 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ and $24 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, respectively. Of course, the medium required for

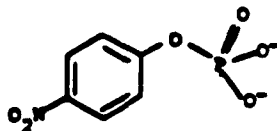
*Calculated from the reported second-order rate constant of $5.70 \text{ M}^{-1} \text{ min}^{-1}$ and pK_a of 7.99 for 2-PAM.¹⁹

solubility is such that a strict comparison cannot be made and future complexes will be designed to overcome the solubility and lability²⁶ problems of these phosphine-based ligands. Nevertheless, it appears that related complexes with ionizable pendants could provide attractive catalysts for facilitating the hydrolysis of phosphate and phosphonate esters.

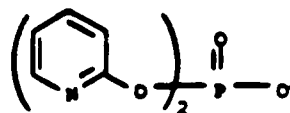
Since January 1985, we have been investigating another area of catalysis of phosphate ester hydrolysis, namely, the ability of 3:Co²⁺ and 4:Co²⁺ to catalyse the hydrolysis of phosphate mono- and diesters. Over the course of a 3 month period, we looked at a variety of esters such as 17-19 and attempted to demonstrate catalysis of their hydrolysis under a variety of



17



18



19

conditions of pH, concentration of catalyst and temperature. The invariable result was that under no circumstances could either metal complex be demonstrated to be active toward these anionic phosphates. Prolonged reaction times were complicated by ligand instability, as was seen in the above study.

III. Literature Cited

1. (a) "Molecular Properties and Mechanism of Alkaline Phosphatase", J.E. Coleman and J.F. Chlebowski in "Advances in Inorganic Biochemistry", G.L. Eichorn and L.G. Marzli, Eds., Elsevier, North Holland, (1979), Vol. 1., pp 1-66. (b) "Alkaline Phosphatase: An Enzyme With Multiple Catalytic Metal Ions at Each Active Center: ^{31}P and ^{113}Cd NMR in Solution Correlated With the Crystal Structure". J.E. Coleman and P. Gettins, *Rev. Port. Quím.*, (1985), 27, 33. (c) "Alkaline Phosphatase, Solution Structure, and Mechanism", J.E. Coleman and P. Gettins in "Advances in Enzymology", A. Meister, Ed., Wiley Interscience, New York, (1983), 55, pp 381-452. (d) "The Three Dimensional Structure of Alkaline Phosphatase from E. Cole", H.W. Wyckoff, M. Handschumacher, H.M. Krishna Murthy, and J.M. Sowadski in "Advances in Enzymology", A. Meister, Ed., Wiley Interscience, New York, (1983), 55, pp 453-480.
2. (a) "Characterization of the Histidine Residues in Alkaline Phosphatase by C-13 Nuclear Magnetic Resonance", J.D. Otvos and D.T. Browne, Biochemistry, (1980), 19, 4011. (b) "Characterization of the Properties of the Multiple Metal Binding Sites in Alkaline Phosphatase by C-13 Nuclear Magnetic Resonance", J.D. Otvos and I.M. Armitage, Biochemistry, (1979), 19, 4021.
3. "Models for Zn(II) Binding Sites in Enzymes", R.S. Brown, N.J. Curtis, and J. Huguet in "Metal Ions in Biological Systems", H. Sigel, Ed., Marcel Dekker, Basel (1982), 16, 55 and references therein.
4. "Oxonase and Esterase Activities of Erthrocyte Carbonic Anhydrase", Y. Pocker and S. Sarkanen, Biochemistry, (1978), 17, 1110.
5. (a) "Enzymatic Hydrolysis of Organophosphorus Compounds, VI. Effect of Metallic Ions on the Phosphorylphosphatases of Human Serum and Swine Kidney", K.B. Augustinsson and G. Heimbürger, Acta Chem. Scand., (1955), 9, 383. (b) "Enzymatic Hydrolysis of Organophosphorus Compounds I. Occurrence of Enzymes Hydrolysing Dimethylamidoethoxyphosphoryl Cyanide (Tabun)", K.B. Augustinsson and G. Heimbürger, Acta Chem. Scand., (1954), 8, 753. (c) "Nature of the Serum Enzyme Catalysing Paraoxon Hydrolysis", D.E. Lenz, L.E. Suquehery, and J.E. Holton, Biochem. Biophys. Acta, (1973), 321, 189. (d) "Purification of a DFP (diisopropylphosphorofluoridate)-hydrolysing Enzyme From Squid Head Ganglion", F.C.G. Hoskin and R.J. Long, Arch. Biochem. Biophys., (1972), 150, 148.
6. (a) "Carbonic Anhydrase Models 5. Tris(4,5-di-n-propyl-2-imidazolyl)phosphine- Zn^{2+} and Bis(4,5,diiso-propyl-2-imidazolyl)-2-imidazolylphosphine- Zn^{2+} . Catalysts

- Facilitating $\text{HCO}_3^- \rightleftharpoons \text{CO}_2$ Interconversion", H. Slebocka-Tilk, J.L. Cocho, Z. Frakman, and R.S. Brown, J. Am. Chem. Soc., (1984), **106**, 2421. (b) "Tris(imidazole)-Containing Phosphine: M^{2+} Complexes as Biomimetic Catalysts. Importance of a $\text{L}:\text{M}^{2+}-\text{OH}$ in the Catalysed Biomolecular Hydrolysis of *p*-Nitrophenylpicolinate", R.S. Brown, M. Zamkane, and J.L. Cocho, J. Am. Chem. Soc., (1984), **106**, 5222.
7. (a) "Mechanisms of Nucleophilic Substitution in Phosphate Esters", J.R. Cox and O.B. Ramsay, Chem. Rev., (1964), **64**, 317. (b) "Insecticides (VI). Rate of Hydrolysis of Some H_3PO_4 Esters", J.A. Ketelaar and A.R. Gersmann, Rec. Trav. Chem., (1958), **77**, 973.
 8. "Phosphatübertyrung mit Salzen von α -Pyridylphosphorsäuren", W. Kamp, Chem. Ber., (1965), **98**, 1038.
 9. "Structure and Anticholinesterase Activity of a Series of Ethyl Substituted Phenyl Methyl Phosphonates", A.M. Relimpio, Z. Naturforsch., (1977), **32C**, 760.
 10. (a) "Catalysis by Hydrophobic Tetraalkylammonium Ions. Dephosphorylation of *p*-Nitrophenyl Diphenyl Phosphate". C.A. Bunton, Y.S. Hong, L.S. Romsted, and C. Quan, J. Am. Chem. Soc., (1981), **103**, 5781 and references therein. (b) "Dephosphorylation in Functionalized Micelles. The Role of the Imidazole Group". J.M. Brown, C.A. Bunton, S. Diaz, and Y. Ihara, J. Org. Chem., (1980), **45**, 4169 and references therein.
 11. "Remarkable Activation of Anionic Nucleophiles Toward *p*-Nitrophenylacetate by Aqueous Trioctylmethylammonium Chloride: A New Class of the Hydrophobic Aggregate", Y. Okahata, R. Ando, and T. Kunitake, J. Am. Chem. Soc., (1977), **99**, 3067.
 12. (a) "Micellar Effects Upon Dephosphorylation and Deacylation by Oximate Ions". C.A. Bunton and Y. Ihara, J. Org. Chem., (1977), **42**, 2865. (b) "Micellar Acceleration of Organophosphate Hydrolysis by Hydroximinomethylpyridinium Type Surfactants". J. Epstein, J.J. Kaminski, N. Bodor, J. Sowa, and T. Higuchi, J. Org. Chem., (1978), **43**, 2816. (c) "Nucleophilic Cleavage of Phosphate Triesters in Dialkylammonium Bilayer Membranes". Y. Okahata, H. Ihara, and T. Kunitake, Bull. Chem. Soc., (1981), **54**, 2072.
 13. (a) "Hydrolysis of Di- and Trisubstituted Phosphate Esters Catalysed by Nucleophilic Surfactants". C.A. Bunton and L.G. Ionescu, J. Am. Chem. Soc., (1973), **95**, 2912. (b) "The Hydrolysis of *p*-Nitrophenyl Diphenyl Phosphate". C.A. Bunton, S.J. Farber, and E.J. Fendler, J. Org. Chem., (1968), **33**, 29.

14. "Imidazole-Promoted Hydrolysis of Oxaphospholene Esters. Nucleophilic vs. General Base Catalysis". R.S. Macombe, J. Am. Chem. Soc., (1983), 105, 4386.
15. "Solvolysis of Tetrabenzyl Pyrophosphate. Catalysis by Imidazole". R. Blakeley, F. Kerst, and F.H. Westheimer, J. Am. Chem. Soc., (1966), 88, 112.
16. (a) "A Cyclic Phosphate: Base and Metal Acetate Catalysed Ring Opening". W.G. Wadsworth and W.S. Wadsworth, J. Am. Chem. Soc., (1983), 105, 1631 and references therein. (b) "Lewis Acid Catalysed Methanolysis of a Phosphate Triester", W.S. Wadsworth, J. Org. Chem. (1981), 46, 4080. (c) "Base Hydrolysis of the Penta-ammine(trimethylphosphate)iridium Ion", P. Hendry and A.M. Sargeson, J. Chem. Soc., Chem. Commun., (1984), 164.
17. "Cobalt (III) Complex Catalysed Hydrolysis of Phosphorus Esters", R.A. Kenley, R.H. Flemming, R.M. Laine, D.S. Tse, and J.S. Winterle, Inorg. Chem., (1984), 23, 1870 and references therein.
18. "The Hydrolysis of Phosphonate Esters", R.F. Hudson and L. Keay, J. Chem. Soc., (1956), 2463.
19. From data reported in "Nonquaternary Cholinesterase Reactivators. 2. α -Heteroaromatic Aldoximes and Thiohydroximates as Reactivators of Ethyl Methylphosphonyl-Acetylcholinesterase in Vitro", R.A. Kenley, C.D. Bedford, O.D. Dailey, R.A. Howd, and A. Miller, J. Med. Chem., (1984), 27, 1201.
20. "Design, Synthesis and Study of Catalysts for Organophosphate Ester Hydrolysis". R.S. Brown, Annual Summary Report Contract No. DAMD17-83-C-3091, (1984).
21. "E.D.T.A. Titrations". H.A. Flaschka, 2nd Ed., Pergamon Press, New York, 1964.
22. (a) "Reactivities of *p*-Nitrophenyl Esters of Phosphorus Acids in Alkaline Hydrolysis", W.E. Bel'skii, L.A. Kudryavtseva, O.M. Il'ina, and B.E. Ivanov, Zh. Obshch. Khim., (1979), 49, 2470. (b) "The Chemistry of Phosphorus", J. Emsley and D. Hall, J. Wiley, New York (1976), 494.
23. (a) "Hydrolysis of Phosphate Esters Bound to Co(III). Kinetics and Mechanism of Intramolecular Attack of Hydroxide on Coordinated 4-Nitrophenyl Phosphate", D.R. Jones, L.F. Kindoy, and A.M. Sargeson, J. Am. Chem. Soc., (1983), 105, 7327. (b) "Geometrical and Stereochemical Factors in Metal-Promoted Amide Hydrolysis", J.T. Groves and R.R. Chambers, J. Am. Chem. Soc., (1984), 106, 630.

24. (a) "Mixed Metal Complexes as Enzyme Models. I. Intra-Complex Nucleophilic Catalysis by an Oxime Anion", R. Breslow and D. Chipman, J. Am. Chem. Soc., (1965), 87, 4195. (b) "An Artificial Enzyme Combining a Metal Catalytic Group and a Hydrophobic Cavity", R. Breslow and L.E. Overman, J. Am. Chem. Soc., (1970), 92, 1075. (c) "Models for Metalloenzymes. The Zn(II)-Catalysed Transesterification of N-(β -Hydroxyethyl)ethylenediamine by p-Nitrophenylpicolinate", D.S. Sigman and C.T. Jorgensen, J. Am. Chem. Soc., (1972), 94, 1724. (d) "Metalloenzyme Models. Divalent Metal Ion Catalysed Hydrolysis of p-Nitrophenylpicolinate in the Presence of Imidazoles and Pyridines Having Hydroxyl Groups in Their Side Chains", K. Ogino, K. Shindo, T. Minami, W. Tayaki, and T. Eiki, Bull. Chem. Soc. Jpn., (1983), 56, 1101. (e) "Models for Metalloenzymes. Zn(II)-ion Catalysed Phosphorylation of 1,10-Phenanthroline-2-carbinol by Adenosine Tri-Phosphate", D.S. Sigman, G.M. Wahl, and D.J. Creighton, Biochemistry, (1972), 11, 2236.
25. (a) "Hydrolysis of Pyrophosphate to Orthophosphate Promoted by Co(III). Evidence for the Role of a Polynuclear Species", P.W. Hübner and R.M. Milburn, Inorg. Chem., (1980), 19, 1267 and references therein. (b) "Solvolysis of Organic Phosphates. IV. 3-Pyridyl and 8-Quinolyl Phosphates as Effected by the Presence of Metal Ions", Y. Murakami and J. Sunamoto, Bull. Chem. Soc. Jpn., (1971), 44, 1827. (c) "Magnesium Ion Catalysis of Hydrolysis of Isopropyl Methylphosphonofluoridate. The Charge Effect in Metal Ion Catalysis", J. Epstein and W.A. Mosher, J. Phys. Chem., (1968), 72, 622. (d) "Metal Ion Activation of Phosphate Transfer by Bidentate Coordination", F.J. Farrell, W.A. Kjellstrom, and T.G. Spiro, Science, (1969), 164, 320.
26. "Zn²⁺-Catalyzed Decomposition of a Tri(imidazol-2-yl) phosphine. Crystal and Molecular Structure of [Bis(4,5-diisopropylimidazol-2-yl)phosphinic acid]dichlorozinc Hydrate". R.G. Ball, R.S. Brown, and J.L. Cocho, Inorg. Chem. (1984), 23, 2315.

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